FORM PTO-1390 REV. 2/01T — U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371

ATTORNEY'S DOCKET NUMBER
08685.0001
Customer No.: 22,852

U.S. APPLICATION NO. (If known, see 37CFR1.5)
09/980819

INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/PT00/00007	June 9, 2000	June 9, 1999

TITLE OF INVENTION: **PRODUCTION BY YEASTS OF ASPARTIC PROTEINASES FROM PLANT ORIGIN WITH SHEEP'S, COW'S, GOAT'S MILK, ETC. CLOTTING AND PROTEOLYTIC ACTIVITY**

APPLICANT(S) FOR DO/EO/US: **1) Maria Salomé SOARES PAIS, 2) Filomena da Conceição S. S. CALIXTO, and 3) Rudy J. PLANTA**

Applicants herewith submit to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☐ This is an express request to begin national examination procedures (35 U.S.C. 371(f)). The submission must include items (5), (6), (9) and (21) indicated below.
4. ☒ The US has been elected by the expiration of 19 months from the priority date (Article 31).
5. ☒ A copy of the International Application as filed (35 U.S.C. 371 (c)(2)).
   a. ☒ is attached hereto (required only if not communicated by the International Bureau.
   b. ☒ has been communicated by the International Bureau.
   c. ☐ is not required, as the application was filed with the United States Receiving Office (RO/US).
6. ☐ An English language translation of the International Application as filed (35 U.S.C. 371 (c)(2)).
   a. ☐ is attached hereto.
   b. ☐ has been previously submitted under 35 U.S.C. 154 (d)(4).
7. ☒ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371 (c)(3)).
   a. ☐ are attached hereto (required only if not communicated by the International Bureau).
   b. ☐ have been communicated by the International Bureau.
   c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
   d. ☒ have not been made and will not be made.
8. ☐ An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371 (c)(3)).
9. ☐ An oath or declaration of the inventor(s) (35 U.S.C. 371 (c)(4)).
10. ☒ Annexes of the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371 (c)(5)).

Items 11 to 20 below concern document(s) or information included:

11. ☒ Information Disclosure Statement under 37 CFR 1.97 and 1.98
12. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
13. ☒ A FIRST preliminary amendment.
14. ☐ A SECOND or SUBSEQUENT preliminary amendment.
15. ☐ A Substitute specification.
16. ☐ A change of power of attorney and/or address letter.
17. ☐ A computer-readable form of the sequence listing in accordance with PCT Rule 13ter.2 and 35 U.S.C. 1.821-1.825.
18. ☐ A second copy of the published international application under 35 U.S.C. 154 (d)(4).
19. ☐ A second copy of the English language translation of the international application 35 U.S.C. 154 (d)(4).
20. ☒ Other items or information:
   a. ☒ Copy of cover page of International Publication No. WO 00/75283.
   b. ☐ Copy of Notification of Missing Requirements.

U.S. APPLICATION NO. (If known, see 37CFR 1.5) 09/980819	INTERNATIONAL APPLICATION NO. PCT/PT00/00007	ATTORNEY'S DOCKET NUMBER: 08685.0001

21. ☒ The following fees are submitted:

BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)):

Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO **$1040.00**

International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO **$890.00**

International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search fee (37 CFR 1.445(a)(2)) paid to USPTO **$740.00**

International preliminary examination fee (37 CFR 1.482) paid to USPTO but all claims did not satisfy provisions of PCT Article 33(1)-(4) **$710.00**

International preliminary examination fee (37 CFR 1.482) paid to USPTO nd all claims satisfied provisions of PCT Article 33 (1)-(4) **$100.00**

	CALCULATIONS PTO USE ONLY	
ENTER APPROPRIATE BASIC FEE AMOUNT =	$890.00	
Surcharge of $130.00 for furnishing the oath or declaration later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492 (e)).	$	

CLAIMS	NUMBER FILED		NUMBER EXTRA	RATE		
Total Claims	26	- 20 =	6	x $18.00	$54.00	
Independent Claims	5	-3 =	2	x $84.00	$168.00	
MULTIPLE DEPENDENT CLAIM(S) (if applicable)				+$280.00	$280.00	
				TOTAL OF THE ABOVE CALCULATIONS =	$1392.00	
☐ Applicant claims small entity status. See 37 CFR 1.27. The fees indicated above are reduced by ½.					$696.00	
				SUBTOTAL =	$696.00	
Proccssing fee of **$130.00** for furnishing the English translation later than ☐ 20 ☐ 30 months from the earliest priority date (37 CFR 1.492(f)).					$	
				TOTAL NATIONAL FEE =	$696.00	
Fee for recording the enclosed assignment (37 CFR 1.21 (h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). **$40.00** per property. +					$	
				TOTAL FEES ENCLOSED =	$696.00	
					Amount to be refunded:	$
					charged:	$

a. ☒ A check in the amount of $ 696.00 to cover the above fees is enclosed.

b. ☐ Please charge my Deposit Account No. _____ in the amount of $ _____ to cover the above fees. A duplicate copy of this sheet is enclosed.

c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 06-0916. A duplicate copy of this sheet is enclosed.

d. ☐ Fees are to be charged to a credit card. **WARNING:** Information on this form may become public. **Credit card information should not be included on this form.** Provide credit card information and authorization on PTO-2038.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137 (a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

Finnegan, Henderson, Farabow, Garrett & Dunner, L.L.P.
1300 I Street, N.W.
Washington, D.C. 20005-3315 DEC 07 2001

SIGNATURE

Ernest F. Chapman/25,961

PATENT
Attorney Docket No. 08685.0001
CUSTOMER NUMBER 22,852

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:

Maria Salomé SOARES PAIS et al.

Serial No.: Not Yet Assigned

Filed: December 7, 2001

National Stage of International Application No. **PCT/PT00/00007** under 35 U.S.C. 371, for **PRODUCTION BY YEASTS OF ASPARTIC PROTEINASES FROM PLANT ORIGIN WITH SHEEP'S, COW'S, GOAT'S MILK, ETC. CLOTTING AND PROTEOLYTIC ACTIVITY**

Group Art Unit: Not Yet Assigned

Examiner: Not Yet Assigned

Assistant Commissioner for Patents
Washington, DC 20231

Sir:

PRELIMINARY AMENDMENT

Prior to examination, please amend the above-identified application as follows:

IN THE SPECIFICATION:

Please amend the specification as follows:

Page 1, after the title insert a new paragraph as follows:

--CROSS REFERENCE TO RELATED APPLICATIONS

This application is national phase application based on PCT/PT00/00007, filed June 9, 2000, and claims the priority of Portuguese Patent Application No. 102318 B, filed on June 9, 1999.--

FINNEGAN
HENDERSON
FARABOW
GARRETT &
DUNNER LLP

1300 I Street, NW
Washington, DC 20005
202.408.4000
Fax 202.408.4400
www.finnegan.com

IN THE CLAIMS:

Please amend claims 1, 11, and 19 as follows:

1. (Amended) A method for producing an aspartic proteinase from plant origin using yeast as a host cell said method comprising the introducing into that host cell a plant DNA construct containing the sequence encoding said aspartic proteinase from plant origin and growing said host cell comprising said plant DNA construct containing the sequence encoding said aspartic proteinase from plant origin in a culture medium whereby said aspartic proteinase from plant origin or part thereof is secreted or not into the culture medium.

11. (Amended) A cell according to Claims 9 or 10 wherein said aspartic proteinase is a plant aspartic proteinase or a part thereof.

19. (Amended) A method for detection of the aspartic proteinase from plant origin either in the cell extracts or in the culture medium using the polyclonal antibodies against plant-origin acidic aspartic proteinase (CCMP1).

REMARKS

Applicants have amended claims 1, 11, and 19 to improve clarity. A marked up version of the amended claims is attached and captioned "Appendix Detailing Amendments to the Claims Pursuant to 37 C.F.R. §1.21(c)(1)(ii)." No new matter has been introduced by these amendments.

The examiner is respectfully requested to consider the above preliminary amendment prior to examination of the application.

FINNEGAN
HENDERSON
FARABOW
GARRETT &
DUNNER LLP

1300 I Street, NW
Washington, DC 20005
202.408.4000
Fax 202.408.4400
www.finnegan.com

PATENT
Attorney Docket No. 08685.0001
CUSTOMER NUMBER 22,852

If there are any fees due in connection with the filing of this Preliminary Amendment, please charge the fees to Deposit Account No. 06-0916.

Respectfully submitted,

FINNEGAN, HENDERSON, FARABOW, GARRETT & DUNNER, L.L.P.

Dated: December 7, 2001

By: _____
Ernest F. Chapman
Reg. No. 25,961

EFC/FPD/sci

FINNEGAN
HENDERSON
FARABOW
GARRETT &
DUNNER LLP

1300 I Street, NW
Washington, DC 20005
202.408.4000
Fax 202.408.4400
www.finnegan.com

APPENDIX DETAILING AMENDMENTS TO THE CLAIMS PURSUANT TO 37 C.F.R. 1.121 (c)(1)(ii)

CLAIM AMENDMENTS

Applicants submit the following claims marked up for the Examiner's convenience to show changes as required by rule 37 C.F.R. 1.121 (c)(1)(ii). This paper is not intended to be entered.

1. (Amended) A method for producing an aspartic proteinase from plant origin using yeast as a host cell said method comprising the introducing into that host cell a plant DNA construct containing the sequence encoding [the] said aspartic proteinase from plant origin and growing said host cell comprising said plant DNA construct containing the sequence encoding said aspartic proteinase from plant origin in a culture medium whereby said aspartic proteinase from plant origin or part thereof is secreted or not into the culture medium.

11. (Amended) A cell according to Claims 9 [and] or 10 wherein said aspartic proteinase is a plant aspartic proteinase or a part thereof.

19. (Amended) A method for detection of the aspartic proteinase from plant origin either in the cell extracts or in the culture medium using the [antibody CCMP1] polyclonal antibodies against plant-origin acidic aspartic proteinase (CCMP1).

FINNEGAN
HENDERSON
FARABOW
GARRETT &
DUNNER LLP

1300 I Street, NW
Washington, DC 20005
202.408.4000
Fax 202.408.4400
www.finnegan.com

09/980819
JC10 Rec'd PCT/PTO 0 7 DEC 2001
ART 34 AMDT

DESCRIPTION

Production by yeasts of aspartic proteinases from plant origin with sheep's, cow's, goat's milk, etc. clotting and proteolytic activity.

BACKGROUND OF THE INVENTION

Field of the Invention

The use of a yeast expression system has become a way of producing large quantities of different types of compounds on an industrial scale. Regarding the production of plant-origin aspartic acid proteinases with industrial applications, there has not been any news of yeast expression with regard to production for use on an industrial scale.

The object of this invention patent, described below, refers to the construction of plasmids, the transformation of yeast strains and the production of plant-origin aspartic acid proteinases. These proteins are proteolytic and milk clotting enzymes which can be used in the cheese production and other biotechnological applications

Description of the Prior Art

Plant aspartic proteinases have been isolated, characterised and cDNA have been prepared since 1997 (D'Hondt et al, 1997). The studies with the aspartic proteinases derived from *Cynara cardunculus* named Cyprosins started in the nineties, with the purification of the enzymes (at that time known as Cynarases, Heimgartner *et al*, 1990), followed in 1992 with their partial characterisation. The construction of a cDNA library and the isolation of a cDNA clone was first reported in 1993 (Cordeiro 1993) and published in several journals since 1994, together with the characterisation of their tissue specificity (Brodelius *et al*, 1995; Cordeiro *et al*, 1994; 1994; Cordeiro *et al*, 1995). The sequence of the CYPRO11 cDNA was included in the gene bank and reported later on (Brodelius *et al*, 1998). Purification of Cardosins, the other group of *Cynara cardunculus* aspartic proteinases, was achieved in 1995 (Faro *et al*, 1995). After this, an extensive work was performed with respect to some biochemical properties including specificity towards substrates (Faro *et al*, 1995; Veríssimo *et al*,1995, 1996). Characterisation and partial protein sequence analysis started in 1995 (Faro *et al*, 1995; Veríssimo *et al*, 1996). Since then, the studies performed in further characterisation of the enzymes, their glycosylation pattern (Costa *et al*, 1997), their

histological and cytological location (Ramalho-Santos *et al*, 1997) and function (Faro *et al*, 1998) have been published. The characterisation of the enzyme precursor (Ramalho-Santos *et al*, 1998a) and identification of its proteolytic processing mechanism (Ramalho-Santos *et al*, 1998b) helped to understand the molecular and physiological relevance of the intra-molecular domains such as the pro-sequence and the plant-specific-insert. Crystallisation studies on the structure of Cardosin A started in 1998 (Bento *et al*, 1998) and has contributed to the knowledge of intramolecular aspects related to the biological function (Frazão *et al*, 1999). Only very recently the cDNA encoding the Cardosin A was cloned. Functional aspects of protein domains and motifs and further implications in the function of this enzyme were better clarified (Faro *et al*, 1999).

The description of a DNA construct for expression of polypeptides by yeast cells was prior reported (EP 0123289). The constructs employed the entire yeast ∝-factor secretion leader. Since then the production of several polypeptides of interest have been reported in yeast cells, including aspartic proteinases from animal origin, as for example bovine chymosin (Mellor et al, Gene 1983, 24: 1-14), and human cathepsin E (Yamada et al, Biochimica et Biophysica Acta 1994, 1206: 279-285).

EXPERIMENTAL

Construction of Plasmids. Transformation of Yeast Strains and Production of Plant Aspartic Proteinases

The insertion of coding gene CYPRO11 into a plant-origin proteinase constitutes the experimental model for controlling the yeast expression of plant-origin aspartic acid enzymes.

Two *Escherichia coli*-yeast expression system vectors were constructed, using a type 2μ multi-copy plasmid and a centromeric plasmid having a low number of copies. The choice of gene used was the leucine deficient one (LEU2). The expression cassette contained developer Gal7 promotor and four different leader sequences upstream from the heterologous gene. Transcription of the heterologous gene was stopped by a PGK1 terminator.

From the different leader sequences tested (native prosequence, preSUC2-proCYPRO11, preMFα-proCYPRO11 and preproMFα), we concluded that preMFα-proCYPRO11 was the best leader sequence for the production of plant-origin aspartic acid proteinases, whether cyprosins corresponding to the plant-origin model proteins coded by gene CYPRO11, or other commercially interesting plant-origin acidic aspartic proteinases.

The MFα yeast presequence is sufficient to develop secretion of the aspartic acid proteinase into the culture medium, and the use of a prosequence of the gene is not necessary. The native prosequence was essential to the active protein's production.

The use of centromeric plasmids having a low number of copies gave better results than type 2μ multi-copy plasmids.

Different yeast strains were tested, including *Saccharomyces cerevisae* BJ1991 (MATα *leu2 trpl ura3-52 prbl-1122 pep4-3*), BJ2168 (MATα *leu2 trpl ura3-52 prcl-1122 pep4-3*), MT302/1c-a (*arg5-6 leu2-12 his3-11 his3-15 peb4-3 ade1*), W303-1^a (*MATα leu2-3,112 ura3-1 trpl-1 his3-11,15 ade2-1 can1-100* GAL SUC2).

These strains were kept on YPD agar plates containing 1% yeast extract, 2% bacto-peptone, 2% glucose and 1.5% agar.

The transformed yeast was grown in an SD medium (0.67% yeast nitrogen base without amino acids, DIFCO, 2% (w/v) glucose), supplemented with amino acids suited to the auxotrophic needs of each strain, except for the leucine one.

The cultures were collected and washed once with sterile distilled water. The cells were resuspended in a YPGal medium (1% yeast extract, 2% bacto-peptone, 4% galactose) and used to inoculate the same medium at a density of A_{600} = 0.2. The cultures were incubated in the same culture conditions until they reached densities of A_{600} = 2, 6 or 10.

Of the yeast strains tested, protease deficient strain BJ1991 produced and secreted into the culture medium the largest quantities of aspartic acid proteinase with considerable milk clotting and proteolytic activity. The secretion of proteolytic enzymes was therefore dependent on culture growth. The recombinant proteinase

with the highest degree of clotting and proteolytic activity was obtained in the stationary phase of the YPGal medium's growth (A_{600} = 10). In the exponential phase (A_{600} = 2), the yeast cells secreted an inactive recombinant proteinase having a high molecular weight. It was considered to be an unprocessed form of the proteinase in which a specific region of the genes of plant-origin acidic aspartic proteinases called a specific plant insert had not been removed.

The largest sub-unit of the recombinant proteinases secreted by the yeast was glycosilated, in the only site possible for glycosilation, and contained a considerable number of manose type glycan chains.

Preparation of Polyclonal Antibodies

The total proteic extract used to produce polyclonal antibodies against plant-origin acidic aspartic proteinase with considerable coagulation and proteolytic activity was obtained from the dry flowers of *Cynara cardunculus* by maceration in a mortar in liquid nitrogen and extraction with 50mM of Tris HCI buffer at a pH of 8.3 (Heimgartner et al., 1990). The proteins were fractionated in 12% SDS-PAGE using 100µg of total protein extract per well. The gel was tinted with a 0.02% Commassie Blue solution in distilled water. The bands corresponding to the largest sub-unit of the plant enzyme (31-32.5kDa in the SDS-PAGE gel) were isolated and the content of each well was sent to EUROGENTEC (Belgium) for the production of antibodies.

Isolation of the Plant-origin Proteinase and Western Blotting Analysis

Isolation of the recombinant plant-origin proteinase from the cell extracts was done using 30ml of yeast cells grown to densities of A_{600} = 2, 6 or 10. After collection, the cells were washed with distilled water, resuspended in 500µl of buffer and exploded by shaking them with glass balls.

Isolation of the recombinant proteinase from the culture medium was done after collecting the medium and concentrating it almost 10 times by ultracentrifugation.

The proteinase concentration was ascertained using the Bio-Rad protein analysis kit in accordance with the manufacturer's instructions. 50µg of total proteic extract from the yeast cells or 1.125g of the concentrated culture medium was analysed in 12%

SDS-PAGE. The proteins were transferred to a nitro-cellulose membrane (Bio-Rad) using Trans-Blot SD Semi-Dry Electrophoretic Transfer Cell (Bio-Rad) equipment in accordance with the manufacturer's instructions. Proteins were detected using polyclonal antibody CCMPI prepared in accordance with the description in the previous section and Boeringer Mannheim's Chemiluminescence Western Blotting Kit in accordance with the manufacturer's instructions.

The results obtained showed that the transformed yeast produces plant-origin aspartic acid proteinase and that the inactive form is found in cells in the exponential growth phase while the active form is secreted into the culture medium. This peculiarity is decisive when it comes to getting good performance for the extraction and purification of plant-origin acidic aspartic proteinases produced from yeast.

Analysis of the Plant-origin Recombinant Enzyme's Clotting and Proteolytic Activity

Proteolytic activity was analysed in accordance with the Twinning method (1984). The casein preparation marked with isothiocyanate (casein-FTC) was made in accordance with the author's instructions. The reactive mixture contained 30µl of 0.2M sodium citrate buffer, pH 5.1, 20l of casein-FTC and 20µl of enzyme solution (3µg/µl in the case of total proteic extract from the yeast cells or 150ng/µl in the case of concentrated culture medium).

Two control tests were done by replacing the enzymatic solution with the reactive buffer. Another control was performed by using the same yeast strain transformed with the same plasmids in which the heterologous gene was absent. The samples were incubated at 37°C for 30 minutes. Reaction was stopped by adding 120µl of 5% trichloracetate acid (TCA) in all but one of the controls. In the latter case, the same amount of 0.5M Tris HCI buffer at a pH of 8.0 (positive control) was added. The samples were centrifuged and a 150µl aliquot of the supernatant fraction was diluted to 3ml with 0.5M Tris HCI buffer at a pH of 8.5. The control (without enzymes), whose reaction was stopped with the TCA solution, was used to ascertain the formation of soluble fluorescent compounds in TCA with enzyme intervention. Relative fluorescence of the samples was ascertained using wavelengths of 490nm for excitation and 525nm for emission in a Shimadzu RF-1501 (Shimadzu Corporation,

Kyoto, Japan) spectrofluorimeter. The percentage of relative fluorescence (%RF) was calculated by subtracting the negative control values from the values, and considering the positive control values as 100%RF. For statistical analysis of the results, each sample had three replicas and three independent readings were taken. The data obtained were analysed with the Student's *t* test (α=0.05). Greatest proteolytic activity, obtained for the best combination/yeast strain, was 15% RF/μ of protein. This figure refers to standard culture conditions, and can be increased under conditions optimised for industrial purposes namely using mutant yeast strains chosen for their maximum recombinant proteinase secretion into the culture medium.

Ascertaining Clotting Activity

Clotting activity was ascertained in test tubes, using unconcentrated culture medium in accordance with the following method: 10ml of the culture medium of the transformed YPGal yeast cells was added to 3ml 12% of skimmed milk (bacto-Difco) and 100ml mM $CaCl_2$. The pH of the culture medium for the culture grown to either A_{600} = 6 or 10 was approximately 5.0. For the culture medium of the culture grown to A_{600} = 2, the pH was adjusted to 5.0 using HCl. The samples were kept at 37°C until the onset of coagulation. The coagulation was evident.

CLAIMS

1. A method for producing an aspartic proteinase from plant origin using yeast as a host cell said method comprising the introducing into that host cell a DNA construct containing the sequence encoding the said aspartic proteinase from plant origin and growing said host cell comprising said DNA construct containing the sequence in a culture medium whereby said aspartic proteinase from plant origin or part thereof is secreted or not into the culture medium

2. A method according to Claim 1 whereby said DNA sequence forms part of a DNA construct which is introduced into said host cell and which comprises in the direction of transcription a pro sequence heterologous to said host cell or to said aspartic proteinase from plant origin and said pro-sequence is joined in reading frame to the said DNA sequence coding for the mature aspartic proteinase from plant origin whereby said aspartic proteinase from plant origin is secreted by said host cell

3. A method according any one of Claims 1 and 2 wherein said aspartic proteinase from plant origin is a plant enzyme

4. A method according to 1 to Claim 3, wherein said enzyme is a plant aspartic proteinase or an unprocessed form thereof

5. A method according to Claim 1 to 4 wherein said enzyme is cyprosin or mutant forms thereof

6. A method according to any one of the Claims 1 to 4 wherein said aspartic proteinase from plant origin is cardosin or mutant forms thereof

7. A method according to any one of Claims 1 to 6 wherein said host cell is an yeast strain with laboratory or industrial interest

8. A method according to any one of Claims 1 to 7 wherein said host cell is from the genus *Saccharomyces* used for the transformation and expression of plant aspartic proteinases encoding genes and the secretion of the aspartic proteinase from plant origin encoded by said genes or secretion of part of said aspartic proteinase from plant origin

9. A transformed yeast host cell comprising an expression cassette which comprises, in the direction of transcription a leader sequence functional in said host cell composed of a pro-sequence heterologous to said host cell or to an aspartic proteinase from plant origin and said pro-sequence is joined in reading frame to the DNA sequence encoding for the said mature aspartic proteinase from plant origin

10. A cell according to Claim 9 wherein said pro-sequence is a plant aspartic proteinase pro-sequence

11. A cell according to Claims 9 and 10 wherein said aspartic proteinase is a plant aspartic proteinase or a thereof

12. A cell according to Claim 9, 10 and 11 wherein said aspartic proteinase from plant origin is cyprosin or an unprocessed form thereof

13. A cell according to Claim 9, 10, 11 and 12 wherein said aspartic proteinase from plant origin is cardosin or an unprocessed form thereof

14. The expression cassettes constructs for use in a yeast host cells comprising: in the direction of transcription a leader sequence composed of a pro-sequence heterologous to said host cell or to aspartic proteinase from plant origin and said pro-sequence is joined in reading frame to the DNA sequence encoding for the said mature aspartic proteinase from plant origin

15. The expression cassettes constructs according to Claim 14, wherein said pro-sequence is heterologous to said host cell or to said aspartic proteinase from plant origin or to said host cell and said aspartic proteinase from plant origin

16. The expression cassettes constructs according to Claim 14 or 15 further comprising the pro-sequence of the plant aspartic proteinase and the plant gene encoding plant aspartic proteinases

17. A method according to any one of Claims 1 to 8 wherein said aspartic proteinase from plant origin or part thereof is isolated either from the cell extracts or from the culture medium

18. A method for detection of the aspartic proteinase from plant origin either in the cell extracts or in the culture medium using the antibody raised against the said aspartic proteinase from plant origin

19. A method for detection of the aspartic proteinase from plant origin either in the cell extracts or in the culture medium using the antibody CCMP1

20. The transformed yeast cells in culture described in Claims 9 to 13 characterised by their production of recombinant plant aspartic proteinases with milk clotting activity which cleave caseins from milk of different origins, namely sheep's, cow's and goat's milk confirmed by milk clotting tests

21. The transformed yeast cells in culture described in Claims 9 to 13 characterised by their production of recombinant plant aspartic proteinases including cyprosins and cardosins capable of giving to cheese a special taste and flavour

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
14 December 2000 (14.12.2000)

PCT

(10) International Publication Number
WO 00/75283 A1

(51) International Patent Classification[7]: C12N 1/19 // 15/57

(21) International Application Number: PCT/PT00/00007

(22) International Filing Date: 9 June 2000 (09.06.2000)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
102318 B 9 June 1999 (09.06.1999) PT

(71) Applicant *(for all designated States except US)*: **INSTITUTO DE CIENCIA APLICADA E TECNOLOGIA (ICAT)** [PT/PT]; Edificio ICAT, Campo Grande, P-1449-016 Lisboa (PT).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **SOARES PAIS, Maria, Salomé** [PT/PT]; Edificio ICAT - Campo Grande, P-1749-016 Lisboa (PT). **CALIXTO, Filomena da Conceição, S., S.** [PT/PT]; Rua8 de Março, Lote 11, P-2860 Moita (PT). **PLANTA, Rudy, J.** [NL/NL]; Dep. of Biochemistry and Molecular Biology, De Boelelaan 1083, Amsterdam (NL).

(74) Agent: **RIBEIRO CARDOSO, Vitor, Luis**; Rua Morgado de Setúbal, 65, 3 Dtº, P-2910-672 Setúbal (PT).

(81) Designated States *(national)*: AE, AU, BR, CA, CH, CN, DE, DK, DM, DZ, ES, FI, GB, IL, LU, MA, NZ, PT, TR, US, ZA.

(84) Designated States *(regional)*: European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published:
— *With international search report.*
— *Before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments.*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 00/75283 A1

(54) Title: PRODUCTION BY YEASTS OF ASPARTIC PROTEINASES FROM PLANT ORIGIN WITH SHEEP'S, COW'S, GOAT'S MILK, ETC. CLOTTING AND PROTEOLYTIC ACTIVITY

(57) Abstract: This invention is valid for recombinant enzymes produced from transformed yeast with coding genes for plant-origin aspartic acid proteinases. These proteinases have considerable sheep's, cow's and goat's milk clotting activity. They can be produced in large quantities by cultivating transformed yeast in a liquid medium. They are secreted into the culture medium and can be supplied in liquid or lyophilised form. The activity of these enzymes is similar to that of chymosin (an animal-origin enzyme) used in the production of cheese on an industrial scale. Recombinant aspartic acid proteinases differ from chymosin in their casein cleavage capacity. Recombinant plant enzymes cleave α, β and κ caseins. Chymosin only cleaves κ casein. The ability of plant-origin recombinant aspartic acid proteinases to cleave α, β and κ caseins is responsible for the special flavour, smell and consistency of the cheese produced.

Customer Number 22,852
Attorney Docket No 08685.0001

DECLARATION AND POWER OF ATTORNEY

As a below named inventor, I hereby declare that: my residence, post office address and citizenship are as stated below next to my name; I believe I am the original, first, and sole inventor (if only one name is listed below) or an original, first, and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

PRODUCTION BY YEASTS OF ASPARTIC PROTEINASES FROM PLANT ORIGIN WITH SHEEP'S, COW'S, GOAT'S MILK, ETC. CLOTTING AND PROTEOLYTIC ACTIVITY

the specification of which
☐ is attached and/or
☒ was filed on **December 7, 2001** as United States Application Serial No. **09/980,819** and was amended on December 7, 2001 or
☒ PCT International Application No. **PCT/PT00/0007**, filed on **June 9, 2000**, and was amended on **November 8, 2001**.

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above. I acknowledge the duty to disclose information which is material to patentability as defined in 37 CFR § 1.56.

I hereby claim foreign priority benefits under 35 U.S.C. § 119(a)-(d) or § 365(b) of any foreign application(s) for patent or inventor's certificate or § 365(a) of any PCT International application(s) designating at least one country other than the United States, listed below and have also identified below, any foreign application(s) for patent or inventor's certificate, or any PCT International application(s) having a filing date before that of the application(s) of which priority is claimed:

Country	Application Number	Date of Filing	Priority Claimed Under 35 U.S.C. 119
PORTUGAL	102318 B	June 9, 1999	☒ YES ☐ NO
			☐ YES ☐ NO

I hereby claim the benefit under 35 U.S.C. § 119(e) of any United States provisional application(s) listed below:

Application Number	Date of Filing

I hereby claim the benefit under 35 U.S.C. § 120 of any United States application(s) or § 365(c) of any PCT International application(s) designating the United States, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT International application(s) in the manner provided by the first paragraph of 35 U.S.C. § 112, I acknowledge the duty to disclose information which is material to patentability as defined in 37 CFR § 1.56 which became available between the filing date of the prior application(s) and the national or PCT International filing date of this application:

Application Number	Date of Filing	Status (Patented, Pending, Abandoned)

I hereby appoint the following attorney and/or agent(s) to prosecute this application and transact all business in the Patent and Trademark Office connected therewith. **FINNEGAN, HENDERSON, FARABOW, GARRETT & DUNNER, L.L.P.**, CUSTOMER NUMBER 22,852, Douglas B. Henderson, Reg. No. 20,291; Ford F. Farabow, Jr., Reg. No. 20,630; Arthur S. Garrett, Reg. No. 20,338; Donald R. Dunner, Reg. No. 19,073; Brian G. Brunsvold, Reg. No. 22,593; Tipton D. Jennings, IV, Reg. No. 20,845; Jerry D. Voight, Reg. No. 23,020; Laurence R. Hefter, Reg. No. 20,827; Kenneth E. Payne, Reg. No. 23,096; Herbert F. Mintz, Reg. No. 26,691; C. Larry O'Rourke, Reg. No. 26,014; Albert J. Santorelli, Reg. No. 22,610; Michael C. Elmer, Reg. No. 26,857; Richard H. Smith, Reg. No. 20,609; Stephen L. Peterson Reg. No. 26,325; John M. Romary, Reg. No. 26,331; Bruce C. Zotter, Reg. No. 27,680; Dennis P. O'Reilley, Reg. No. 27,932; Allen M. Sokal, Reg. No. 26,695; Robert C. Bajefsky, Reg. No. 25,387; Richard L. Stroup, Reg. No. 28,478; David W. Hill, Reg. No. 28,220; Thomas L. Irving, Reg. No. 28,619; Charles E. Lipsey, Reg. No. 28,165; Thomas W. Winland, Reg. No. 27,605; Basil J. Lewris, Reg. No. 28,918; Martin I. Fuchs, Reg. No. 28,508; E. Robert Yoches, Reg. No. 30,120; Barry W. Graham, Reg. No. 29,924; Susan Haberman Griffen, Reg. No.

30,907; Richard B. Racine, Reg. No. 30,415; Thomas H. Jenkins, Reg. No. 30,857; Robert E. Converse, Jr., Reg. No. 27,432; Clair X. Mullen, Jr., Reg. No. 20,348; Christopher F. Foley, Reg. No. 31,354; John C. Paul, Reg. No. 30,413; Roger D. Taylor, Reg. No. 28,992; David M. Kelly, Reg. No. 30,953; Kenneth J. Meyers, Reg. No. 25,146; Carol P. Einaudi, Reg. No. 32,220; Walter Y. Boyd, Jr., Reg. No. 31,738; Steven M. Arzalone, Reg. No. 32,095; Jean B. Fordis, Reg. No. 32,984; Barbara C. McCurdy, Reg. No. 32,120; James K. Hammond, Reg. No. 31,964; Richard V. Burgujian, Reg. No. 31,744; J. Michael Jakes, Reg. No. 32,824; Thomas W. Banks, Reg. No. 32,719; Christopher P. Isaac, Reg. No. 32,616; Bryan C. Diner, Reg. No. 32,409; M. Paul Barker, Reg. No. 32,013; Andrew Chanho Sonu, Reg. No. 33,457; David S. Forman, Reg. No. 33,694; Vincent P. Kovalick, Reg. No. 32,867; James W. Edmondson, Reg. No. 33,871; Michael R. McGurk, Reg. No. 32,045; Joann M. Neth, Reg. No. 36,363; Gerson S. Panitch, Reg. No. 33,751; Cher M. Taylor, Reg. No. 33,216; Charles E. Van Horn, Reg. No. 40,266; Linda A. Wadler, Reg. No. 33,218; Jeffrey A. Berkowitz, Reg. No. 36,743; Michael R. Kelly, Reg. No. 33,921; James S. Monroe, Reg. No. 33,971; Doris Johnson Hines, Reg. No. 34,629; Allen R. Jensen, Reg. No. 28,224; Lori Ann Johnson, Reg. No. 34,498; and David A. Manspeizer, Reg. No. 37,540 and . Please address all correspondence to **FINNEGAN, HENDERSON, FARABOW, GARRETT & DUNNER, L.L.P.**, 1300 I Street, N.W., Washington, D.C. 20005, Telephone No. (202) 408-4000.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Full Name of First Inventor	Inventor's Signature	Date
Maria Salomé Soares Pais		18/05/2002
Residence Av. Rainha D. Amélia 28, 6.º Esq.º Lisboa, Portugal 1600-039 Lisboa, Portugal PTX		Citizenship Portugal
Post Office Address Laboratório de Biotecnologia Vegetal (ICAT) Edifício ICAT - Campo Grande 1749-016 Lisboa, Portugal		

Full Name of Second Inventor	Inventor's Signature	Date
Filomena da Conceição Sousa Soares Calixto		18/05/2002
Residence RUA DR. SILVA EVARISTO 80, 2º ESQº Moita, Portugal PTX 2860-462 MOITA, PORTUGAL		Citizenship Portugal
Post Office Address R. Dr. Silva Evaristo, nº 80, 2º Esq.º 2860-462, Moita, Portugal		

Full Name of Third Inventor	Inventor's Signature	Date
Rudy J. Planta (Deceased) By **(Keetie Henriette Ouborg Planta - Legal Representative)**	(Legal Representative)	4-05-2002
Residence Amsterdam, The Netherlands (of the Deceased at Time of Death) NLX Amsterdam, The Netherlands (Legal Representative) NLX		Citizenship Netherlands (Deceased) Netherlands (Legal Representative)
Post Office Address (of the Deceased at Time of Death) Dept. of Biochemistry and Molecular Biology De Boelelaan 1083 Amsterdam, The Netherlands (Legal Representative) Mezquitalaan 40 1064 NS Amsterdam The Netherlands		